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Augmented hyperaemia and reduced tissue injury in response to ischaemia in subjects with the 34C > T variant of the *AMPD1* gene

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Aims In patients with coronary artery disease, the 34C > T variant of the adenosine mono-phosphate deaminase gene (*AMPD1*), encoding a dysfunctional protein, predicts improved survival. We hypothesized that in subjects with this variant allele, ischaemia-induced intracellular adenosine formation is increased, augmenting reactive hyperaemia and ischaemic tolerance.

Methods and results We selected 10 healthy subjects with the CT genotype and 10 CC controls. The forearm vasodilator response to 2 and 5 min of ischaemia (venous occlusion plethysmography, expressed as percentage of maximum blood flow after 13 min of ischaemia) was higher in the CT group 56% (49–74%) and 77% (71–86%) vs. 49% (42–53%) and 60% (55–70%) in the CC group [median (interquartile range), $P = 0.01$]. Additionally, ischaemia–reperfusion injury was assessed in the thenar muscle using ^{99m}Tc-annexin A5 scintigraphy after forearm ischaemic exercise to detect externalized membrane phosphatidylserines. At reperfusion, ^{99m}Tc-annexin was administered intravenously. The change in annexin targeting between 1 and 4 h post-injection was –2.3% (interquartile range –2.4 to –1.6%) in the CT group vs. –0.3% (–0.6 to 1.3%) in controls ($n = 7$ in both groups, $P = 0.03$).

Conclusion The 34C > T variant of *AMPD1* augments vasodilation and reduces tissue injury in response to forearm ischaemia. These mechanisms could contribute to the survival benefit of cardiovascular patients with this variant allele.

Introduction

In patients with heart failure and in those with coronary artery disease, the presence of the 34C > T polymorphism on at least one of the alleles encoding for the enzyme adenosine mono-phosphate deaminase (*AMPD1*) is associated with prolonged survival.^{1,2} AMPD is one of the enzymes involved in the intracellular metabolism of the purine nucleoside adenosine (Figure 1), catalysing the conversion of AMP to IMP.³ In humans, four AMPD isoforms have been described, named after the source from which they were initially purified: M (muscle), L (liver), E1, and E2 (erythrocytes), encoded by the different genes *AMPD1*, *AMPD2*, and *AMPD3*.⁴ Approximately 15–20% of Caucasian and African-American individuals are heterozygous or homozygous for

the 34C > T variant of *AMPD1*, encoding a truncated protein with the loss of function.⁵

Under conditions of impending tissue danger, such as ischaemia, the extracellular adenosine concentration rapidly increases.⁶ Subsequent stimulation of adenosine receptors induces various effects such as vasodilation, ischaemic preconditioning, and inhibition of inflammation.⁷ On the basis of the observation that AMPD activity is significantly reduced in patients heterozygous for the 34C > T variant (CT genotype), it was suggested that in these patients during ischaemia, AMP is preferentially degraded into adenosine, which will increase ischaemic tolerance.¹

We hypothesized that subjects with the 34C > T variant show augmented reactive hyperaemia and reduced ischaemia–reperfusion injury in response to ischaemia. To test this hypothesis, we selected healthy subjects with the CT genotype and controls homozygous for the common C allele (CC genotype), with otherwise similar parameters of adenosine transport and metabolism. The vasodilator response

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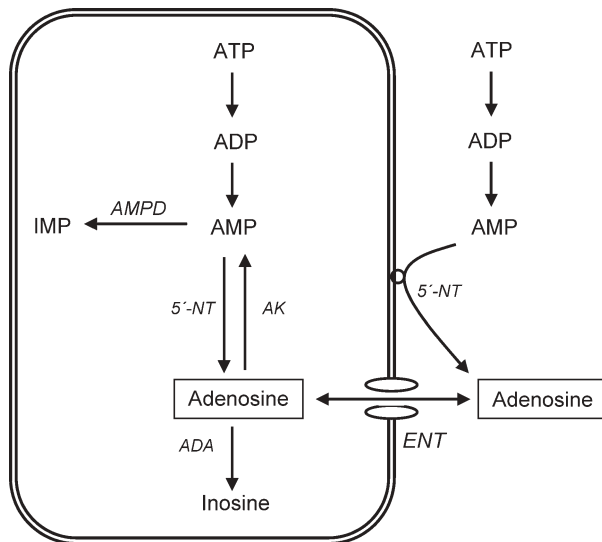


Figure 1 Schematic representation of the formation, transport, and degradation of adenosine. ADA, adenosine deaminase; AK, adenosine kinase; IMP, inosine mono-phosphate; 5'-NT, 5'-nucleotidase.

to 2, 5, and 13 min of forearm ischaemia was determined by venous occlusion plethysmography. During the last minute of the 13 min period of ischaemia, the subjects also performed rhythmic contractions of the hand, because this stimulus has previously been demonstrated to induce maximum vasodilation, which is dependent only on the structural characteristics of the vessel wall.⁸ We hypothesized that subjects with the 34C > T variant of *AMPD1* have higher forearm blood flow (FBF) responses to 2 and 5 min of ischaemia, but not to 13 min of ischaemia. This experiment was repeated in the presence of dipyridamole, as a pharmacological tool to inhibit facilitated diffusion of adenosine through the equilibrative nucleoside transporter (ENT), to explore the role of increased intracellular adenosine formation. We hypothesized that dipyridamole potentiates reactive hyperaemia, as previous evidence suggests that during ischaemia, transport of adenosine over the cellular membrane is mainly from outside the cell inwards.^{9,10} This effect of dipyridamole, however, is expected to be less pronounced in subjects with the CT genotype, because in these subjects, impaired conversion of AMP to IMP will enhance the intracellular adenosine formation, thus decreasing the transmembranous adenosine concentration gradient, which is the driving force for adenosine transport.¹¹

In an additional series of experiments, ischaemia-reperfusion injury was determined in the thenar muscle, using ^{99m}Tc-annexin A5 scintigraphy to detect phosphatidylserine exposure on cellular membranes of affected cells as early marker of ischaemic injury, as described previously.¹²

Methods

Subjects

The study protocol was approved by the Institutional Review Board of the Radboud University Nijmegen Medical Centre. Ninety-six healthy subjects participated after signing informed consent. Blood was drawn for genotyping of the *AMPD1* and adenosine A_{2A} receptor genes (*ADORA2A*) and for the determination of the activities of the ENT, adenosine kinase, and adenosine deaminase. The *ADORA2A* 1976C > T genotype was determined, as the TT variant

could potentially affect its function.¹³ From these volunteers, subjects were selected to participate in the current study or another study on the effect of the 1976C > T polymorphism in *ADORA2A*. Twenty subjects had the CT genotype and two the TT genotype for *AMPD1*. To study reactive hyperaemia, we selected 10 subjects with the CT genotype and 10 control subjects with the CC genotype, with comparable sex, age, and body mass index. To assess tolerance to ischaemia-reperfusion injury, we selected seven male subjects with the CT genotype and seven controls (Table 1).

Post-occlusive reactive hyperaemia

Experiments were performed in a temperature-controlled room (23°C) in the morning after an overnight fast and 24 h of caffeine abstinence. The brachial artery of the non-dominant arm was cannulated with a 20-gauge catheter for intra-arterial drug administration and blood pressure recording. FBF was recorded simultaneously on both arms by venous occlusion plethysmography using mercury-in-silastic strain gauges.¹⁴ Before each recording, a wrist cuff was inflated to 200 mmHg to exclude the hand circulation. Arterial occlusion of the non-dominant forearm was induced by inflation of an additional upper arm cuff to 200 mmHg.

Thirty minutes after insertion, FBF was recorded for 5 min during saline infusion (baseline FBF). Subsequently, the FBF response to 2, 5, and 13 min of forearm ischaemia was measured for 3, 5, and 5 min, respectively, as described previously.¹⁵ During the last minute of the 13 min period of ischaemia, the subjects performed rhythmic handgripping, as a stimulus for maximal forearm vasodilation.⁸ After 40 min of equilibration, this experiment was repeated, but now during concomitant administration of dipyridamole (Persantin[®], Boehringer Ingelheim, Espana SA, Spain) into the brachial artery (7.4 nmol/min/dL of forearm tissue). A previous study demonstrated that this dose does not affect baseline FBF, but significantly potentiates adenosine-induced vasodilation.¹⁶ Administration of dipyridamole was discontinued 10 s after initiation of forearm occlusion and restarted 10 s before reperfusion. After 30 min of equilibration, the FBF response to intrabrachial administration of sodium nitroprusside (SNP; 0.06 and 0.6 µg/min/dL; Pharmacy Radboud University Nijmegen Medical Centre) and acetylcholine (ACh; 0.5 and 2.0 µg/min/dL; miocol[®]-E, Novartis, Bournonville Pharma BV, The Hague, The Netherlands) was recorded for 5 min per dose to study endothelium-independent and endothelium-dependent vasodilation, respectively.

Ischaemia-reperfusion injury

Circulation to the non-dominant forearm was interrupted for 10 min by inflation of an upper arm cuff to 200 mmHg. Concomitantly, subjects performed rhythmic isometric handgripping with a dynamometer at 50% of maximum force, alternating 5 s of contraction and 5 s of relaxation until exhaustion. Immediately on reperfusion, ^{99m}Tc-annexin A5 was administered intravenously into the dominant arm. At 1 and 4 h post-injection, both hands were scanned simultaneously until ≥150 000 counts were detected (or when a maximum scanning time of 25 min was reached) using a gamma camera equipped with a low-energy high-resolution collimator (Siemens Orbiter, Hoffman Estates, IL, USA) connected to a Hermes Gold image processing system (Nuclear Diagnostics, Stockholm, Sweden) as previously described.¹²

Genetic analysis

Blood was drawn in EDTA-containing vacutainers and stored at -70°C until DNA isolation. Genomic DNA isolation was performed using a standard desalting protocol.¹⁷ Genotyping was performed by pyrosequencing according to the protocol of the manufacturer (Pyrosequencing AB, Uppsala, Sweden).¹⁸

For genotyping of the *AMPD1* 34C > T variant, the following reaction was carried out: primers FW8076 (5'-gcaatcatcatgtgtctacc-3', 10 pmol) and RV8077 (5'-agcgcgtctccggtcatagattatagcc

Table 1 Baseline characteristics of the two groups in both studies [median (interquartile range)]; there are no significant differences between the CT and CC groups

Characteristics	Plethysmography study		Scintigraphy study	
	CT genotype	CC genotype	CT genotype	CC genotype
Number (male/female)	10 (7/3)	10 (7/3)	7 (7/0)	7 (7/0)
Age (years)	22 (21–25)	23 (22–25)	24 (21–28)	22 (20–26)
Body mass index (kg/m ²)	22 (20–24)	22 (21–24)	24 (20–25)	22 (20–24)
SBP (mmHg)	126 (118–131)	128 (120–130)	128 (118–136)	128 (128–128)
DBP (mmHg)	68 (66–77)	72 (67–80)	76 (70–92)	72 (62–80)
Heart rate (b.p.m.)	71 (68–76)	76 (64–77)	68 (60–76)	72 (60–76)
Plasma glucose (mmol/L)	4.9 (4.3–5.3)	4.9 (4.1–5.3)	5.1 (4.7–5.5)	4.9 (4.3–5.3)
Cholesterol (mmol/L)	4.4 (3.5–5.2)	4.1 (3.6–4.6)	4.0 (3.2–4.6)	4.1 (3.8–4.5)
Plasma caffeine (mg/L)	0.0 (0.0–0.5)	0.0 (0.0–0.2)	0.1 (0.0–0.2)	0.0 (0.0–0.1)
Uridine uptake				
<i>K_m</i> (μM)	169 (153–201)	171 (158–213)	202 (160–207)	167 (158–211)
<i>V_{max}</i> (nmol/min/mg)	269 (233–311)	286 (261–306)	306 (217–319)	277 (253–298)
AK activity				
<i>K_m</i> (μM)	0.41 (0.36–0.54)	0.48 (0.42–0.62)	0.53 (0.40–0.54)	0.56 (0.49–0.80)
<i>V_{max}</i> (nmol/min/mg)	24 (20–38)	26 (19–35)	25 (21–34)	33 (19–35)
ADA activity				
<i>K_m</i> (μM)	44 (40–47)	44 (40–47)	42 (40–47)	45 (39–46)
<i>V_{max}</i> (nmol/min/mg)	70 (46–104)	66 (56–76)	74 (53–95)	66 (51–73)
ADORA2A 1976 genotype	9 CT/1 CC	9 CT/1 CC	3 CT/3 CC/1 TT	4 CT/3 CC

atgtttctgaatta-3', 1 pmol) were combined with a biotinylated universal primer (4205: 5'-Biotin-gctgctccggttcatagatt-3', 9 pmol), 50 ng of DNA, 0.32 mM dNTPs, 0.5 U Taq DNA polymerase (Invitrogen, Breda, The Netherlands), and a PCR buffer containing 60 mM Tris-HCl pH 8.5, 15 mM ammonium sulfate, and 1.5 mM MgCl₂ in a total volume of 25 μL. For genotyping of the *ADORA2A* 1976C > T variant, the following amplification reaction was carried out: primers FW8073 (5'-gacggga-caccgctgatcgtttacggagg ccaatggcta-3', 1 pmol) and RV8074 (5'-cc caactgactggctcaag-3', 10 pmol) were combined with a biotin-labelled universal primer (4206: 5'-Biotin-gggacaccgctgatcgttta-3', 9 pmol), 50 ng DNA, 0.32 mM dNTPs, 0.5 U Taq DNA polymerase (Invitrogen), and a PCR buffer containing 10 mM Tris-HCl pH 8.0, 50 mM KCl, 0.1% Triton X-100 (v/v), 0.015% gelatin (w/v), 5% DMSO (v/v), and 1.5 mM MgCl₂ in a volume of 25 μL. The cycling conditions for both amplification reactions were similar, starting with 5 min at 92°C, followed by 39 cycles (35 in the case of *ADORA2A*) of 1 min at 92°C, 1 min at the optimized annealing temperature (55°C for *AMPD1* and 54°C for *ADORA2A*), and 1 min at 72°C, then followed by an extra 5 min at 72°C. The amplifications were performed in a PTC-200 Multicycler (MJ-Research via Biozym, Landgraaf, The Netherlands).

Pyrosequencing for *AMPD1* was performed in a forward assay using primer 8078 (5'-tcatacagctgaagagaaa-3') and that for *ADORA2A* in a reverse assay using primer 8075 (5'-ctcaccagcccca-3').

Laboratory analysis

In isolated erythrocytes, uridine uptake was determined as described previously.¹⁹ *V_{max}* and *K_m* values for the ENT were estimated according to Michaelis-Menten kinetics. For the determination of adenosine deaminase activity, adenosine was added to erythrocyte lysate in Tris-buffer (0.35% erythrocytes) in a final concentration of 25, 50, 100, 200, and 300 μmol/L at 37°C. After 15 min, 50 μL 1.5 M HClO₄ was added, and the solution was placed on ice. After centrifugation, 0.5 M triethylamine in chloroform was added to the supernatant in equal volumes. Finally, after centrifugation, the neutralized supernatant was used for the detection of inosine and hypoxanthine with reversed-phase HPLC using tetrabutylammonium hydrogen sulfate (10 mM) as the ion-pair forming agent (pH 6.0) with UV detection set at 254 nm. The sum of inosine and hypoxanthine was used for the calculation of *V_{max}* and *K_m* for adenosine deaminase.

For the determination of adenosine kinase activity, adenosine was added to 10 μL lysate in 43 μL distilled water, 2 μL 50 mM MgCl₂, 2 μL 100 mM DTT, 2 μL 50 mM GTP, 0.7 μL 3 mM erythro-9-(2-hydroxynon-3-yl)-adenine (EHNA), and 100 μL Tris-buffer in a final concentration of 0.1, 0.2, 0.5, 1, and 2 μmol/L at 37°C. After 3.5 min, 50 μL 1.5 M HClO₄ was added, and the solution was placed on ice. After centrifugation, 100 μL 0.5 M triethylamine in chloroform was added to 150 μL supernatant. Finally, after centrifugation, the AMP concentration in the supernatant was measured with reversed-phase HPLC using tetrabutylammonium hydrogen sulfate (10 mM) as the ion-pair forming agent (pH 7.7) with UV detection set at 260 nm. The increase in AMP was used to calculate *V_{max}* and *K_m* values. Plasma caffeine concentration was determined by reversed-phase HPLC with UV detection set at 273 nm.²⁰ All measurements were performed in duplicate.

Preparation of ^{99m}Tc-HYNIC-annexin A5

^{99m}Tc-HYNIC-annexin A5 was prepared as described previously.²¹ Briefly, recombinant human annexin A5 (kindly provided by Dr Vanderheijden, Theseus Imaging Corporation, Boston, MA, USA) was conjugated with succinimidyl-hydrazinonicotinic acid (HYNIC). About 100 μg samples of the annexin-HYNIC conjugate in 500 μL 113 mM tricine, pH 6.8, were stored at -20°C. Before each experiment, ^{99m}Tc-Pertechnetate (500 MBq) was added to the 100 μg sample of the annexin-HYNIC conjugate in the presence of 50 μg stannous sulfate. About 400 MBq ^{99m}Tc-annexin A5 was administered to each subject.

Statistical analysis

As not all variables showed a Gaussian distribution, we present the data as median and interquartile range, and we used Wilcoxon and Koziol tests to evaluate differences. All tests were two-sided and a result with *P* < 0.05 was considered statistically significant. The primary tests in two different studies were on the difference in vasodilator response and in annexin targeting, respectively. The effect of dipyridamole on reactive hyperaemia in the first study was a secondary endpoint, which only served to support the main findings, and the *P*-values of this analysis should be interpreted

with some caution, as we applied no correction for multiple testing. Analysis of the FBF responses was performed off-line blinded for the specific *AMPD1* genotypes. For each post-occlusive period, the highest FBF was selected. The FBF response to 2 and 5 min of ischaemia was expressed as percentage of maximum FBF after 13 min of ischaemia. A Koziol test was used to compare these responses between both genotype groups. The effect of dipyridamole on FBF responses to 2 and 5 min of ischaemia was expressed as percentage increase, and a Koziol test was used to compare both genotype groups.

In the second study, all digital scintigraphic images were analysed off-line by the same blinded investigator (W.J.G.O.), using Hermes Gold software. Region-of-interest analysis was performed for the thenar muscle region, as described previously.¹² Radioactivity was expressed as counts per pixel. To correct for background activity, the targeting of annexin was expressed as the percentage difference between the experimental and control hand, and the results were expressed as the change in targeting between 1 and 4 h post-reperfusion, as described previously.¹²

Results

V_{\max} and K_m values of adenosine deaminase, adenosine kinase, and the ENT did not differ between both groups, and *ADORA2A* genotype frequencies were equally distributed (Table 1).

Post-occlusive reactive hyperaemia

Baseline FBF did not significantly differ between both groups [2.1 (1.7–2.6) mL/min/dL of forearm tissue in the CT genotype group and 2.2 (1.7–2.9) mL/min/dL in the CC group; $P=0.8$]. In addition, maximum vasodilation in response to 13 min of ischaemia did not differ between the groups [38.5 (31.9–46.0) mL/min/dL in the CT group vs. 40.5 (32.4–51.3) mL/min/dL in the CC group; $P=0.5$]. FBF in response to the 2 and 5 min period of ischaemia was higher in the CT genotype group when compared with the controls (Figure 2, $P=0.01$). There were no differences in FBF in the control arm and mean arterial pressure between both groups.

The effect of administration of dipyridamole on baseline FBF was not different between both groups [from 2.1 (1.4–2.7) to 2.3 (1.4–3.0) mL/min/dL in the CT genotype group

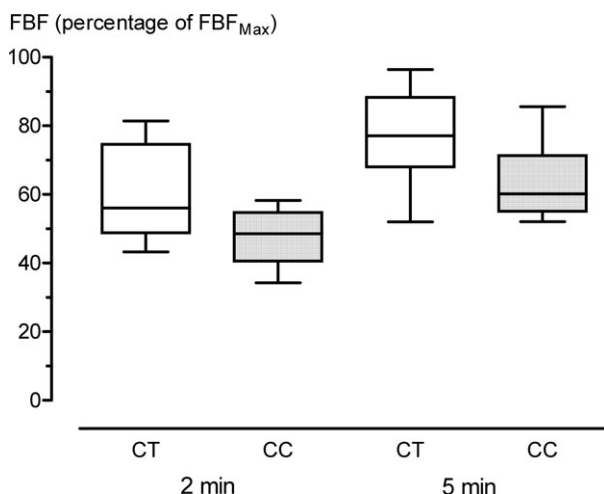


Figure 2 Box plot of the FBF responses to 2 and 5 min of ischaemia, expressed as percentage of maximum FBF. $P=0.01$ for comparison between both groups.

Effect of dipyridamole on FBF (percentage increase)

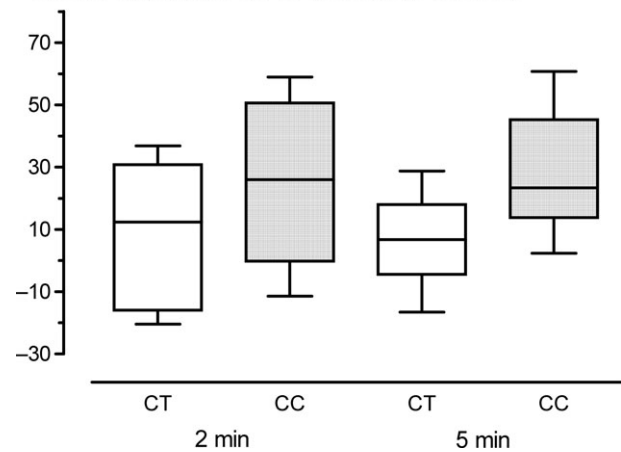


Figure 3 Box plot of the effect of administration of dipyridamole on the FBF responses to 2 and 5 min of ischaemia, expressed as percentage increase when compared with the FBF response without dipyridamole. $P=0.01$ for comparison between both groups.

and from 2.0 (1.4–2.5) to 2.4 (2.0–3.1) mL/min/dL in the CC group; $P=0.4$). Dipyridamole significantly potentiated FBF responses to 2 and 5 min of ischaemia in the control group [FBF 25.2 (19.9–32.4) and 35.5 (31.2–42.1) mL/min/dL during dipyridamole infusion after 2 and 5 min of ischaemia, respectively, $P=0.02$ and 0.005 vs. FBF without dipyridamole], but not in the CT genotype group [FBF 27.4 (21.5–32.3) and 31.9 (29.5–40.0) mL/min/dL during dipyridamole administration after 2 and 5 min of ischaemia, $P=0.2$ and 0.1 vs. FBF without dipyridamole, respectively]. The effect of dipyridamole was more pronounced in the CC genotype group than in the CT group (Figure 3, $P=0.01$).

Finally, SNP- and ACh-induced vasodilation did not differ between both groups. Administration of SNP increased FBF from 2.8 (1.7–3.3) mL/min/dL at baseline to 7.3 (4.6–8.0) and 14.6 (11.5–19.8) mL/min/dL in subjects with the CT genotype and from 2.5 (2.0–3.2) mL/min/dL to 6.4 (5.2–8.7) and 11.6 (8.9–21.7) mL/min/dL in the CC control group ($P=0.8$). Administration of ACh increased FBF from 3.1 (2.4–4.9) mL/min/dL at baseline to 6.8 (3.6–9.1) and 9.9 (5.2–12.3) mL/min/dL in subjects with the CT genotype and from 3.2 (2.7–3.9) mL/min/dL to 6.8 (5.2–11.4) and 8.2 (6.2–15.3) mL/min/dL in the CC control group ($P=1.0$).

Ischaemia–reperfusion injury

There was no difference in the duration of ischaemic exercise [140 (130–190) s in the CT genotype group vs. 170 (125–180) s in the controls, $P=0.7$] and maximum contractile force [50 (46–72) kg in the CT genotype group vs. 48 (40–52) kg in controls, $P=0.3$] between both groups.

Annexin targeting was less in the CT genotype group than in CC controls (Figure 4; $P=0.03$).

Discussion

The results of the present study demonstrate that in subjects with the 34C > T variant of *AMPD1*, reactive hyperaemia in response to forearm ischaemia is enhanced and tissue injury is reduced. These mechanisms could well contribute to the reduced cardiovascular mortality in patients with genotypes containing the variant allele.

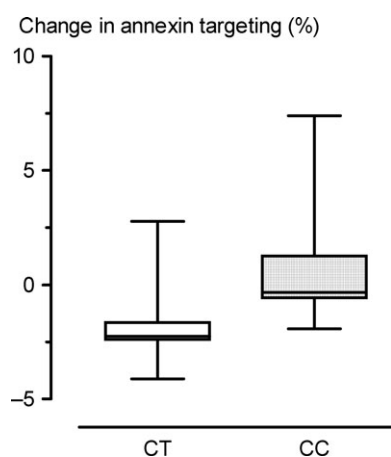


Figure 4 Box plot of the difference in annexin targeting between 1 and 4 h post-reperfusion. $P = 0.03$ for comparison between the groups.

Previous studies have shown that the 34C > T variant of *AMPD1* is associated with improved survival in patients with heart failure¹ and in those with coronary artery disease.² Also in cardiac donors, the 34C > T variant appears to protect against acute heart failure.²² It is unknown which mechanism is responsible for these beneficial cardiovascular effects. The C→T transition results in a nonsense mutation encoding a truncated AMPD protein.⁵ AMPD activity in human muscle biopsies is 30–40% of normal in subjects with the CT genotype and <2% in subjects homozygous for the variant allele.²³ Although predominantly expressed in skeletal muscle, *AMPD1* is also expressed in cardiomyocytes and aorta.³ Indeed, also in cardiac tissue, AMPD activity is ~50% reduced in patients with the CT genotype.²⁴ Considering the various beneficial cardiovascular effects of adenosine receptor stimulation, it is logical to assume that in subjects with AMPD deficiency, ischaemia-induced adenosine formation and subsequent adenosine receptor stimulation are potentiated, provided that alternative routes of adenosine metabolism are unchanged (Figure 1) and that this mechanism is responsible for the improved cardiovascular outcome.

In the present study, we focused on reactive hyperaemia, because previous studies demonstrated that reactive hyperaemia is, at least partially, mediated by adenosine receptor stimulation.²⁵ We postulated that a higher reactive hyperaemia in subjects with the 34C > T variant of *AMPD1* indicates increased adenosine receptor stimulation. In addition, the enhanced reactive hyperaemia itself could also have beneficial effects on recovery after an ischaemic insult.^{26,27} This mechanism was already suggested by recent preliminary data in healthy volunteers with the 34C > T variant, which demonstrated augmented reactive hyperaemia in the forearm, but only in the second minute after reperfusion, and after correction for per cent body fat.²⁸ In line with these observations, we showed that reactive hyperaemia in response to 2 and 5 min of ischaemia is augmented in subjects with the CT genotype, compared with a control group, with otherwise similar activities of adenosine deaminase, adenosine kinase, and the ENT and with similar *ADORA2A* genotype frequencies. The groups did not differ in endothelial function. The observation that reactive hyperaemia in response to a stimulus for maximum forearm vasodilation was similar between both

groups excludes that structural changes of the vessel wall account for the observed differences. Moreover, we corrected for possible structural differences by expressing the FBF responses as percentage of maximum FBF in each subject.

We assume that this augmented reactive hyperaemia is mediated by increased adenosine receptor stimulation. Unfortunately, it is extremely difficult to reliably measure adenosine concentrations in humans *in vivo*, because of the short half-life of adenosine in blood (<1 s)²⁹ and because the endothelium acts as a highly active metabolic barrier for adenosine.³⁰ Therefore, the concentration of adenosine in blood does not reflect that of adenosine in the vicinity of adenosine receptors on several cell types, such as endothelial cells, cardiomyocytes, and vascular smooth muscle cells. Several studies have demonstrated that during strenuous exercise, the tissue adenosine concentration in skeletal muscle biopsies is increased in subjects with the CT genotype when compared with controls, but there are no data on the extracellular adenosine concentration during exercise or ischaemia in these subjects.^{23,31} Because of these difficulties in measuring adenosine concentration, we previously validated dipyridamole as a pharmacological tool to inhibit the ENT. We demonstrated that systemic administration of dipyridamole (administered orally) significantly inhibits *ex vivo* adenosine uptake in isolated erythrocytes.¹⁹ Secondly, during concomitant administration of adenosine into the brachial artery, administration of dipyridamole increased the adenosine concentration in the venous effluent (as measured with microdialysis).³² Thirdly, in the same dose as in the present study, dipyridamole did not affect baseline vascular tone, but significantly potentiated the vasodilator response to the intrabrachial administration of adenosine.³³ Finally, the vasodilator response to intrabrachial administration of dipyridamole and the haemodynamic responses to the systemic administration of dipyridamole could significantly be reduced by adenosine receptor antagonists.^{16,34} In the present study, we used this property of dipyridamole to explore the contribution of facilitated adenosine transport to the observed difference in reactive hyperaemia between subjects with the CT genotype and controls.

It is well known that during ischaemia, the extracellular adenosine concentration rapidly increases.^{6,35} In the present study, dipyridamole potentiated reactive hyperaemia in both groups (although this was not significant for the CT genotype group). This finding indicates that during ischaemia and early reperfusion, flux of adenosine over the cellular membrane is mainly directed inwards. In subjects with the CT genotype, the effect of dipyridamole on maximum reactive FBF was less than in controls. This observation suggests that in these subjects, during ischaemia and early reperfusion, the transmembranous adenosine gradient is less than in control subjects. This is compatible with the view that during ischaemia in subjects with decreased activity of the cytosolic AMPD enzyme, intracellular—and not extracellular—formation of adenosine from AMP is increased.

Adenosine receptor stimulation increases resistance to ischaemia-reperfusion by several mechanisms.³⁶ We hypothesized that in patients with the 34C > T *AMPD1* variant, augmented adenosine formation during ischaemia not only augments reactive hyperaemia, but also reduces ischaemia-reperfusion injury. To test this hypothesis, we used ^{99m}Tc-annexin A5 scintigraphy to determine

ischaemia-reperfusion injury in thenar muscle. We demonstrated that ischaemia-reperfusion injury is attenuated in subjects with the 34C > T genotype. ^{99m}Tc-annexin A5 scintigraphy is based on the high-affinity binding of annexin A5 to phosphatidylserine residues on cellular membranes. It is well documented that loss of membrane asymmetry is an early general feature of apoptosis, resulting in externalization of phosphatidylserine residues in affected cells, thus providing binding sites for annexin A5.³⁷ Alternatively, some previous studies have demonstrated that phosphatidylserine exposure could be a reversible event, preceding commitment to apoptotic cell death.³⁸ By labelling recombinant annexin A5 with ^{99m}technetium, it is possible to detect these cellular changes *in vivo* with a gamma camera.³⁹ In previous studies, we validated ^{99m}Tc-annexin A5 scintigraphy as a sensitive model to detect ischaemia-reperfusion injury in skeletal muscle and demonstrated that this injury could be reduced by the administration of adenosine and dipyridamole prior to the ischaemic insult.^{12,19} Additionally, we demonstrated that the adenosine receptor antagonist caffeine abolishes protection by ischaemic preconditioning.²¹

Several potential limitations of our study should be addressed. First, we only studied subjects who were heterozygous for the 34C > T variant. In subjects with the TT genotype, reactive hyperaemia and ischaemic tolerance could be augmented even more. Secondly, for technical reasons, we did not measure plasma adenosine concentrations. However, the observation that the effect of dipyridamole on reactive hyperaemia was less in subjects with the CT genotype provides evidence that indeed intracellular adenosine formation during ischaemia is enhanced in these subjects. Thirdly, we did not determine activities of endo- and ecto-5'-nucleotidase, which is also important in the formation of adenosine (Figure 1). Finally, we studied the response to ischaemia in the forearm and not in the heart. However, the 34C > T variant of *AMPD1* also reduces AMPD activity in cardiac tissue.²⁴

In conclusion, we demonstrated that the 34C > T variant of the *AMPD1* gene augments post-occlusive reactive hyperaemia and tolerance to ischaemia-reperfusion. The reduced potentiating effect of dipyridamole on reactive hyperaemia in subjects with the CT genotype indicates that the *AMPD1* gene determines the effect of dipyridamole and supports the concept that the transmembranous adenosine concentration gradient during ischaemia is diminished in these subjects, which is compatible with increased intracellular formation of adenosine during ischaemia. Our observations predict that in trials with ENT inhibitors designed to explore the protective effect of endogenous adenosine against ischaemia-reperfusion injury, most benefit is expected in patients without the 34C > T variant allele. The augmented reactive hyperaemia and increased ischaemic tolerance could contribute to the previously observed improved survival in cardiovascular patients with this variant allele and bridge the gap between the rational theoretical concept of adenosinergic cardiovascular protection and previous epidemiological observations.

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References

1. Loh E, Rebbeck TR, Mahoney PD, DeNofrio D, Swain JL, Holmes EW. Common variant in *AMPD1* gene predicts improved clinical outcome in patients with heart failure. *Circulation* 1999;**99**:1422–1425.
2. Anderson JL, Habashi J, Carlquist JF, Muhlestein JB, Horne BD, Bair TL, Pearson RR, Hart N. A common variant of the *AMPD1* gene predicts improved cardiovascular survival in patients with coronary artery disease. *J Am Coll Cardiol* 2000;**36**:1248–1252.
3. Morisaki T, Sabina RL, Holmes EW. Adenylate deaminase. A multigene family in humans and rats. *J Biol Chem* 1990;**265**:11482–11486.
4. Sabina RL, Holmes EW. Myoadenylate deaminase deficiency. In: Scriver CR, Beaudet AL, Sly WS, Vale D. *The Metabolic and Molecular Basis of Inherited Disease*. New York: McGraw-Hill; 2001. p2627–2638.
5. Morisaki T, Gross M, Morisaki H, Pongratz D, Zollner N, Holmes EW. Molecular basis of AMP deaminase deficiency in skeletal muscle. *Proc Natl Acad Sci USA* 1992;**89**:6457–6461.
6. Van Wylen DG, Willis J, Sodhi J, Weiss RJ, Lasley RD, Mentzer RM Jr. Cardiac microdialysis to estimate interstitial adenosine and coronary blood flow. *Am J Physiol* 1990;**258**:H1642–H1649.
7. Rongen GA, Floras JS, Lenders JW, Thien T, Smits P. Cardiovascular pharmacology of purines. *Clin Sci (Lond)* 1997;**92**:13–24.
8. Pedrinelli R, Spessot M, Salvetti A. Reactive hyperemia during short-term blood flow and pressure changes in the hypertensive forearm. *J Hypertens* 1990;**8**:467–471.
9. Van Belle H, Goossens F, Wynants J. Formation and release of purine catabolites during hypoperfusion, anoxia, and ischemia. *Am J Physiol* 1987;**252**:H886–H893.
10. Zemgulis V, Wikstrom G, Henze A, Waldenstrom A, Thelin S, Ronquist G. Nucleoside transport inhibition in ischaemic myocardium results in enhanced taurine efflux. *Eur J Pharmacol* 2001;**411**:143–154.
11. Deussen A, Stappert M, Schafer S, Kelm M. Quantification of extracellular and intracellular adenosine production: understanding the transmembranous concentration gradient. *Circulation* 1999;**99**:2041–2047.
12. Rongen GA, Oyen WJG, Ramakers BP, Riksen NP, Boerman OC, Steinmetz N, Smits P. Annexin A5 scintigraphy of forearm as a novel *in vivo* model of skeletal muscle preconditioning in humans. *Circulation* 2005;**111**:173–178.
13. Alsene K, Deckert J, Sand P, De Wit H. Association between A2A receptor gene polymorphisms and caffeine-induced anxiety. *Neuropsychopharmacology* 2003;**28**:1694–1702.
14. Rongen GA, Ginneken E, Thien T, Lutterman JA, Smits P. Preserved vasodilator response to adenosine in insulin-dependent diabetes mellitus. *Eur J Clin Invest* 1996;**26**:192–198.
15. Bijlstra PJ, den Arend JA, Lutterman JA, Russel FG, Thien T, Smits P. Blockade of vascular ATP-sensitive potassium channels reduces the vasodilator response to ischaemia in humans. *Diabetologia* 1996;**39**:1562–1568.
16. Bijlstra P, van Ginneken EE, Huls M, Van Dijk R, Smits P, Rongen GA. Glyburide inhibits dipyridamole-induced forearm vasodilation but not adenosine-induced forearm vasodilation. *Clin Pharmacol Ther* 2004;**75**:147–156.
17. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988;**16**:1215.
18. Ronaghi M. Pyrosequencing for SNP genotyping. *Methods Mol Biol* 2003;**212**:189–195.
19. Riksen NP, Oyen WJ, Ramakers BP, Van den Broek PH, Engbersen R, Boerman OC, Smits P, Rongen GA. Oral therapy with dipyridamole limits ischemia-reperfusion injury in humans. *Clin Pharmacol Ther* 2005;**78**:52–59.
20. Schreiber-Deturmeny E, Bruguerolle B. Simultaneous high-performance liquid chromatographic determination of caffeine and theophylline for routine drug monitoring in human plasma. *J Chromatogr B Biomed Appl* 1996;**677**:305–312.
21. Riksen NP, Zhou Z, Oyen WJG, Jaspers R, Ramakers BP, Brouwer RMHJ, Boerman OC, Steinmetz N, Smits P, Rongen GA. Caffeine prevents

- protection in two human models of ischaemic preconditioning. *J Am Coll Cardiol* 2006;**48**:700–707.
22. Yacoub MH, Yuen AH, Kalsi KK, Birks EJ, Taegtmeyer A, Barton PJ, Johnson PH, Suzuki K, Smolenski RT. C34T AMP deaminase 1 gene mutation protects cardiac function in donors. *Transplantation* 2004;**77**: 1621–1623.
 23. Norman B, Sabina RL, Jansson E. Regulation of skeletal muscle ATP catabolism by AMPD1 genotype during sprint exercise in asymptomatic subjects. *J Appl Physiol* 2001;**91**:258–264.
 24. Kalsi KK, Yuen AH, Rybakowska IM, Johnson PH, Slominska E, Birks EJ, Kaletha K, Yacoub MH, Smolenski RT. Decreased cardiac activity of AMP deaminase in subjects with the AMPD1 mutation—a potential mechanism of protection in heart failure. *Cardiovasc Res* 2003;**59**:678–684.
 25. Carlsson I, Sollevi A, Wennmalm A. The role of myogenic relaxation, adenosine and prostaglandins in human forearm reactive hyperaemia. *J Physiol* 1987;**389**:147–161.
 26. Hamada S, Nishiue T, Nakamura S, Sugiura T, Kamihata H, Miyoshi H, Imuro Y, Iwasaka T. TIMI frame count immediately after primary coronary angioplasty as a predictor of functional recovery in patients with TIMI 3 reperfused acute myocardial infarction. *J Am Coll Cardiol* 2001;**38**: 666–671.
 27. Rochetaing A, Kreher P. Reactive hyperemia during early reperfusion as a determinant of improved functional recovery in ischaemic preconditioned rat hearts. *J Thorac Cardiovasc Surg* 2003;**125**:1516–1525.
 28. Hand BD, Roth SM, Roltsch MH, Park JJ, Kostek MC, Ferrell RE, Brown MD. AMPD1 gene polymorphism and the vasodilatory response to ischemia. *Life Sci* 2006;**79**:1413–1418.
 29. Moser GH, Schrader J, Deussen A. Turnover of adenosine in plasma of human and dog blood. *Am J Physiol* 1989;**256**:C799–C806.
 30. Nees S, Herzog V, Becker BF, Bock M, Des RC, Gerlach E. The coronary endothelium: a highly active metabolic barrier for adenosine. *Basic Res Cardiol* 1985;**80**:515–529.
 31. Sabina RL, Swain JL, Olanow CW, Bradley WG, Fishbein WN, DiMauro S, Holmes EW. Myoadenylate deaminase deficiency. Functional and metabolic abnormalities associated with disruption of the purine nucleotide cycle. *J Clin Invest* 1984;**73**:720–730.
 32. Riksen NP, van Ginneken EE, Van den Broek PH, Smits P, Rongen GA. In vivo evidence against a role for adenosine in the exercise pressor reflex in humans. *J Appl Physiol* 2005;**99**:522–527.
 33. van Ginneken EE, Rongen GA, Russel FG, Smits P. Diadenosine pentaphosphate vasodilates the forearm vascular bed: inhibition by theophylline and augmentation by dipyridamole. *Clin Pharmacol Ther* 2002;**71**: 448–456.
 34. Smits P, Straatman C, Pijpers E, Thien T. Dose-dependent inhibition of the hemodynamic response to dipyridamole by caffeine. *Clin Pharmacol Ther* 1991;**50**:529–537.
 35. Lasley RD, Hegge JO, Noble MA, Mentzer RM. Evidence that cytosolic and ecto 5'-nucleotidases contribute equally to increased interstitial adenosine concentration during porcine myocardial ischemia. *Basic Res Cardiol* 1999;**94**:199–207.
 36. Headrick JP, Hack B, Ashton KJ. Acute adenosinergic cardioprotection in ischaemic-reperfused hearts. *Am J Physiol Heart Circ Physiol* 2003;**285**: H1797–H1818.
 37. Koopman G, Reutelingsperger CP, Kuijten GA, Keehnen RM, Pals ST, van Oers MH. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood* 1994;**84**:1415–1420.
 38. Hammill AK, Uhr JW, Scheuermann RH. Annexin V staining due to loss of membrane asymmetry can be reversible and precede commitment to apoptotic death. *Exp Cell Res* 1999;**251**:16–21.
 39. Blankenberg FG, Katsikis PD, Tait JF, Davis RE, Naumovski L, Ohtsuki K, Kapiwoda S, Abrams MJ, Darkes M, Robbins RC, Maecker HT, Strauss HW. In vivo detection and imaging of phosphatidylserine expression during programmed cell death. *Proc Natl Acad Sci USA* 1998; **95**:6349–6354.